# Proton conductance by the gramicidin water wire

## Model for proton conductance in the $F_1F_0$ ATPases?

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ABSTRACT The gramicidin channel contains a single strand of water molecules associated through hydrogen bonds. Previous work has shown that channels of similar size are formed by association of transmembrane alpha helices of synthetic leucine-serine peptides. Both types of channels translocate protons with considerable selectivity relative to other cations, and it has been proposed that the selectivity arises by proton "hopping" along hydrogen-bonded chains of water, whereas other cations must cross by ordinary diffusion processes. It is possible that a similar mechanism underlies proton transport in the Fo subunit of the $F_1F_0$ ATP synthase. Using the gramicidin channel as a model, we have tested whether a single strand of water is kinetically competent to translocate protons at a rate sufficient to support known rates of ATP synthesis. We found that the gramicidin channel saturates at ~530 pS of protonic current in 4 M HCl, more than sufficient for typical ATP synthesis rates. It follows that proton diffusion to a putative channel in Fo, rather than the channel itself, may limit ATP synthesis rates.

## INTRODUCTION

The $F_1F_0$ ATP synthases couple proton motive force to ATP synthesis. These enzymes are structurally similar in bacteria, chloroplasts, and mitochondia (Senior, 1988), therefore it is reasonable to believe that the mechanism of proton flux through the $F_0$ channel and coupling of flux to ATP synthesis in $F_1$ are essentially the same in most coupling membranes.

The mechanism of proton flux in the $F_0$ channel is not understood, but some of its characteristics are established: (*a*) the protonic current at maximum turnover rate is about 1200 $H^+$ channel$^{-1}$ $s^{-1}$ in $CF_0CF_1$ of intact chloroplasts (Althoff et al., 1989); (*b*) the $F_0$ channel is highly selective to protons over other monovalent ions ($H^+/Na^+ = 10^7$ in $CF_0$; Althoff et al., 1980); and (*c*) in *Escherichia coli,* mutations in $Arg_{210}$ (Cain and Simoni, 1989), $His_{245}$ and $Ser_{206}$ (Cain and Simoni, 1986) of the *a* subunit, and $Asp_{61}$ of the *c* subunit (Hoppe et al., 1982) block proton flux in Fo and are thought to be buried within the membrane.

Nagle and Morowitz (1978) proposed that hydrogen-bonded amino acid residues of membrane proteins might conduct protons by a hopping mechanism analogous to proton transport in ice and liquid water (Onsager, 1973). Cox et al. (1986) and Senior (1988) have incorporated this concept into models for a network of residues aligned at interfaces between α helices in the Fo membrane sector. However, this hypothesis is not consistent with the observation that $Na^+$ currents can drive ATP synthesis by $F_1F_0$ ATPases in halophilic bacteria (Laubinger and Dimroth, 1989; Boyer, 1988). The logic is that $Na^+$ could not permeate a hydrogen-bonded amino acid network, therefore $Na^+$ permeation of $F_0$ precludes such a network.

An alternative route for proton hopping is a network of hydrogen-bonded water molecules analogous to that in the gramicidin channel (Hoppe and Sebald, 1984). Unlike the amino acid network, a channel containing water could also accommodate $Na^+$ permeation. A plausible site for such a channel in $F_0$ is at interfaces between polar domains of α-helices. For example, Lear and co-workers (1988, 1989) have shown that synthetic polypeptides composed of leucine and serine form α-helices in lipid bilayers that can conduct protons or $Na^+$ when the polypeptides form clusters in the plane of the bilayer. The $F_0$ membrane sector in *E. coli* is thought to include a number of α-helices associated with the *a* subunit, the two *b* subunits, and each copy of ~10 *c* subunits. Two of the putative α-helices in the *a* subunit of *E. coli* contain conserved polar residues (Cox et al., 1986; Cain and Simoni, 1986) that would align vertically along one side of each helix, and each *c* subunit includes two putative α-helices that are believed to play a role in proton transport (Fillingame et al., 1990). Thus, it is plausible that these helices associate into tetramers with the polar residues positioned to form channels containing complete or partial water strands.

In this study we used the gramicidin channel as a model for a putative water wire in $F_0$. The gramicidin

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polypeptide forms 4–5-Å diam π-helices that can hydrogen bond to one another at their N-termini and thereby form a transbilayer channel lined with a single strand of hydrogen-bonded waters (Hladky and Haydon, 1984). This water conducts protons by a hydrogen-bonded chain mechanism similar to that in ice (Levitt, 1984; Hladky and Haydon, 1972). The gramicidin channel therefore provides a tool for testing the plausibility of such a water network in $F_0$.

Specific questions we addressed in this report were:

(*a*) How does proton mobility along a single strand of hydrogen-bonded residues within the membrane domain compare with proton mobility in ice? Nagle and Morowitz (1978) assumed that the two mobilities were approximately equal in their original calculations to determine the kinetic feasibility of a hopping mechanism for proton channels. The gramicidin water wire presents a unique opportunity to test this assumption directly.

(*b*) Is a single strand of hydrogen-bonded waters able to transport protons sufficiently fast to account for proton flux in $F_0$ when proton supply from the bulk phase is unlimited? And if this is true, is proton supply to such a narrow target fast enough at physiological pH ranges?

(*c*) Can a single water strand account for the proton selectivity observed in $F_0$? In chloroplasts, the permeability of $CF_0$ to $H^+$ is $10^7$ times greater than $Na^+$ permeability (Althoff et al., 1989). This could be due to selection at the channel (a sieve) or selection at an internal binding site.

Our results show that a water wire is kinetically competent to support proton transport in $F_0$. The rate limiting step at physiological pH ranges appears to be proton diffusion to the channel, rather than transport within the channel itself. Furthermore, the $F_0$ channel must be much more restrictive to alkali cation permeation than is gramicidin, either due to a partial water strand or to selectivity at an internal binding site.

## MATERIALS AND METHODS

### Bilayer chamber

Our chamber was similar to one used previously (Miller, 1987). A polystyrene cup (02-544, Fisher Scientific Co., Pittsburgh, PA) was inserted into one of two overlapping 1.2-cm diam holes drilled into the top of a Teflon block (3.8 × 2.5 × 2 cm). The aqueous volume in the polystyrene cup (0.8 ml) was connected to the aqueous volume in the adjacent Teflon well (1.7 ml) by a hole drilled in the polystyrene using a 0.4-mm diam bit turned by hand. The polystyrene cups were cleaned by tumbling in a 10% solution of dishwashing detergent for ~30 min, followed by extensive rinsing in distilled water, and finally several volumes of doubly distilled, deionized water. The polystyrene cups were used once then discarded. At the beginning of each day, the Teflon chamber was cleaned by boiling in 50% nitric acid. All glassware used in these experiments was cleaned in the same way.

### Lipids

Stock lipid solutions were prepared by dissolving 30 mg of glyceryl monooleate (GMO; Sigma Chemical Co., St. Louis, MO) or 30 mg cholesterol (recrystallized from ethanol) in 1 ml of 2:1 chloroform/methanol prepared from HPLC-grade solvents (Fisher Scientific Co.). These stocks were stored at −20°C for up to 1 wk before use. Unless otherwise noted, 0.1-ml aliquots of each of the lipid stocks were added to each of two glass vials along with 10 μl of a 100-nM gramicidin D stock (Sigma Chemical Co.) in methanol, and then dried under a stream of nitrogen gas. One of the preparations was redissolved by gentle agitation in 0.1 ml of HPLC-grade decane (Aldrich Chemical Co., Milwaukee, WI) and the other in 0.1 ml of HPLC-grade hexadecane (Fisher Scientific Co.). This gave working solutions that were 30 mg $ml^{-1}$ each in GMO and cholesterol with a gramicidin-to-GMO molar ratio of $1:10^7$.

### Formation of planar bilayers

The hole in the polystyrene cup was dried under a stream of nitrogen gas and then just filled with the decane-lipid solvent using a 000 brush, followed by nitrogen drying for 5 min. The polystyrene cup was then pressed into the Teflon chamber and the two compartments were filled with the appropriate aqueous buffer. Freshly coated AgCl electrodes were placed into each of the chambers, and the complete apparatus was placed in a Faraday cage on a marble table. The electrodes were connected to model 3900 patch clamp amplifier (DAGAN Corp., Minneapolis, MN). To form a bilayer, a small amount of the GMO/cholesterol/hexadecane solution was painted onto the opening by a single pass with a clean 000 brush. Within 30 s, a bilayer formed from the solvent as indicated by an abrupt increase in the initial capacitative transient in response to a 1-m V square wave with a 70-ms period. Bilayers formed readily by this technique were stable for more than 1 h at applied potentials as high as 200 mV.

Channels formed in GMO/hexadecane bilayers and in GMO/cholesterol/hexadecane bilayers produced transient currents that were similar in amplitude and duration, but the bilayers with cholesterol were much more stable. Gramicidin could not be inserted into the GMO/cholesterol/hexadecane bilayers from the aqueous phase, therefore it was added to the solvent before membrane formation.

### Measurement of single channel events

A 59-mV potential was applied between the AgCl electrodes at the beginning of each experiment. Channels began to appear 1–5 min after membrane formation. Unless otherwise noted, the signal was filtered at 500 Hz by a four-pole low-pass Bessel filter and was recorded on a model 5113 storage oscilloscope (Tektronix, Inc., Beaverton, OR). The applied potential was then set at the desired test value, 25 events of 100-ms duration or longer were recorded, and then the polarity was reversed and 25 additional events were recorded. Thus, each data point in this report represents the mean of 50 events unless otherwise stated. Apart from the activation energy experiments, all measurements were made at 22°C.

### Determination of lipid hydrolysis at high HCl concentrations

3 mg each of GMO and cholesterol were combined from chloroform/methanol stocks and dried under nitrogen gas. The lipid was sus-

pended in 0.5 ml of doubly distilled, deionized water, warmed to 50°C for 30 s, and then sonicated for 30 s. The suspensions were brought to 1 ml total volume with water and 10 M HCl to yield final HCl concentrations between 0 and 5 M. Incubation was allowed to take place for up to 1 h when it was stopped by extraction of the lipid into 1 ml of 2:1 chloroform/methanol. The solvent was then spotted onto silica gel plates or onto silica-coated glass rods. The unknowns and standards were eluted using 70:30:1 hexane/diethyl ether/acetic acid. Lipid eluted on the rods was quantified by hydrogen flame ionization using an Iatroscan TH-10 detector (RSS Inc., Costa Mesa, CA).

### Measurement of proton and potassium conductivity after exposure of the membrane to 5 M HCl

A GMO/cholesterol/gramicidin D mixture was incubated in 5 M HCl as described above. The GMO/Gramicidin D ratio was $10^7$:1. After 1 h, the suspension was extracted into chloroform/methanol, dried, and redissolved in hexadecane to yield a 30-mg $ml^{-1}$ solution in both GMO and cholesterol as usual. This solution was used to form bilayers in buffers made of 0.1 M HCl or 2.0 M KCl. In either case, single channel conductance was measured at ±59 mV and at ±200 mV.

## RESULTS

### Gramicidin forms proton-conducting channels in GMO/cholesterol/hexadecane bilayers

Fig. 1 *a* shows a recording of gramicidin channels spontaneously opening and closing in a GMO-cholesterol membrane at 1.0 M HCl and 59 mV applied potential. Channel open times of ~1 s were typical as were steps in protonic current which presumably occur when more than one channel is open at once (Hladky and Haydon, 1972). For high amplitude events as shown in Fig. 1, the standard deviation was <10% of the mean amplitude in HCl solutions above 1.0 M, and <20% of the mean amplitude in HCl solutions of 0.1 M or less. Unless otherwise noted, all values given in this report refer to these dominant, high amplitude channels. We also observed lower amplitude channels in all bilayers that contained gramicidin. These channels were less frequent than the high amplitude channels (typically 10% of the total population), less than one-half the amplitude, and about equal in duration. They occurred in the presence of gramicidin D or purified gramicidin A.

### External access step limits gramicidin proton flux at 0.01 M HCl and below; above 0.1 M HCl, the channel limits proton flux

We wished to know the HCl concentrations where proton flux was limited by the channel rather than by the

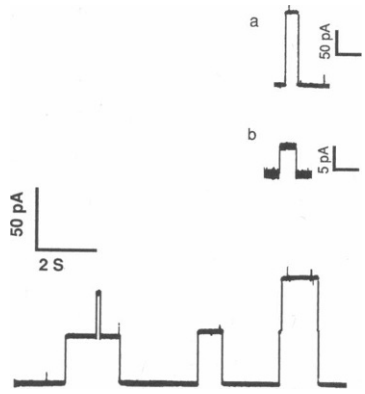


FIGURE 1 Proton currents in single gramicidin D channels. Each step on the oscillscope recording shows spontaneous opening and closing of channels formed by head-to-head gramicidin dimers in 1 M HCl at 59 mV applied potential. Higher steps indicate opening of more than one channel simultaneously. The bilayer membrane in this experiment was formed from glyceryl monooleate (GMO) and cholesterol (1:1 weight ratio) in hexadecane with a gramicidin D/GMO mol ratio of $1/10^7$. The signal was processed at 500 Hz with a four pole, low pass bessel filter. *a* shows a channel from an identical experiment in 0.1 M HCl; *b* is from an experiment in 5 M HCl which was identical except that the gramicidin D/GMO mol ratio was $1/10^8$.

bulk phase. External access steps for ions entering the gramicidin channel appear to be relatively insensitive to applied potential (Andersen, 1983*a*) in agreement with theory (Läuger, 1976). By comparison, ion permeation of the channel itself is very sensitive to applied potential (Hladky and Haydon, 1972; Andersen, 1983*a*) as predicted by some rate models (Läuger, 1973; Nagle, 1987; Hladky, 1987). Thus, current-voltage curves provide a tool for determining the bulk phase HCl concentration at which the rate-limiting step for proton flux shifts from an access step to a step within the channel.

Representative current-voltage curves are shown in Fig. 2. At 0.01 M HCl, the curve is sublinear suggesting that access to the gramicidin channel mouth limits the proton current, whereas at 1.0 M HCl the curve is superlinear suggesting a channel-limited current. Fig. 3 summarizes the entire I-V data set as the ratio of single channel conductances at 200 vs. 59 mV. A ratio of 1 indicates an ohmic I-V relationship, a ratio >1 indicates a superlinear relationship, and a ratio <1 indicates a sublinear relationship. The shift from a ratio <1 at 0.01 M HCl to a ratio >1 at 0.1 M HCl suggests that the transition from an access-limited process to a channel-limited process occurs in that concentration range. This confirms previous results for proton conductance of gramicidin in GMO/hexadecane bilayers (Eisenman et al., 1980).

The current-voltage relationship is an indirect test for the location of rate-limiting steps. As an additional test,

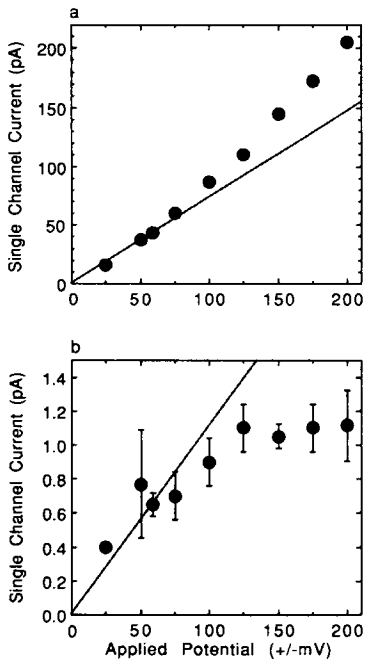


FIGURE 2 The effect of membrane potential on single channel proton currents, (*a*) in 1.0 M HCl, and (*b*) in 0.01 M HCl. Each point represents the mean of 50 events (25 each for positive and negative applied potentials). In *b* the bars represent standard deviations about the means. In *a* the standard deviations were the size of the points or smaller. The lines signify a linear relationship between current and voltage (Ohm's Law) drawn through the points at +/−59 mV.

we compared single channel proton currents in $H_2O$ vs. single channel currents in 90% $D_2O$ + 10% $H_2O$ at 59 mV applied potential. If the rate-limiting step is in the bulk phase, then the isotope ratio for conductance in gramicidin should be similar to that for $H^+/D^+$ conductance in bulk water (1.4; Bockris and Reddy, 1970).

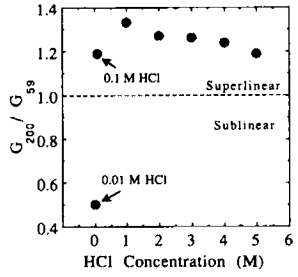


FIGURE 3 The effect of bulk phase HCl concentration on the ratio of proton conductance at 200 mV applied potential ($G_{200}$) to proton conductance at 59 mV applied potential ($G_{59}$). Ratios were calculated from mean channel amplitudes as in Fig. 2. The dashed line represents the $G_{200}/G_{59}$ ratio expected for a circuit that obeys Ohm's Law. Values greater than one indicate a superlinear I–V curve (e.g., Fig. 2 *a*); values less than one indicate a sublinear curve (e.g. Fig. 2 *b*).

Alternatively, a $H^+/D^+$ conductance ratio in gramicidin substantially different from 1.4 would indicate a rate-limiting step associated with the channel. Our results showed that the current ratio for $H_2O/D_2O$ in gramicidin at 0.1 M HCl was 1.34 ± 0.11 which is within experimental error of the ratio measured in liquid water. This was expected based on our current-voltage experiments. At 1.0 M HCl the isotopic current ratio is significantly less at 1.20 ± 0.01. This supports our conclusion that the rate-limiting step in 1.0 M HCl is the gramicidin channel. In 5.0 M HCl (where the channel current is saturated with respect to HCl concentration) the $H^+/D^+$ conductance ratio increases back to 1.35 ± 0.02 suggesting that exit of protons from the terminal water to the *trans* aqueous phase (reverse of the on reaction) may limit the channel current at saturation.

We also tested for a bulk phase-limited step by the addition of sucrose to the medium. Sucrose significantly diminishes the conductivity of aqueous HCl solutions, therefore it would be expected to decrease gramicidin proton currents when proton diffusion to the channel mouth is rate limiting. By comparison, sucrose does not block nor enter the gramicidin channels (Andersen, 1983*a*), and therefore sucrose per se would not be expected to alter flux limited by a channel property. Table 1 shows that addition of 1 M sucrose to 0.01 M HCl decreases the gramicidin proton current as expected from its effect on bulk-phase conductivity. However, 1 M sucrose also decreases the gramicidin proton current at 0.1 M HCl and above (conditions where the current-voltage curves suggested channel-limited flux). Thus, either our conclusions based on the current-voltage relationships and isotope ratios were wrong, or sucrose addition is not a reliable test for an aqueous-diffusion limited process. To distinguish between these possibilities, we measured the effect of 1 M sucrose on $K^+$ flux in 2 M KCl, a condition under which the aqueous diffusion step does not limit $K^+$ flux through the gramicidin channel (Andersen, 1983*b*). Our results show that sucrose significantly diminishes the $K^+$ current in this buffer (Table 1). We conclude that sucrose alters a

**TABLE 1 The effect of 1 M sucrose on proton and potassium currents through the gramicidin channel**

| Electrolyte | Mean single channel current in pA | | | | |
|---|---|---|---|---|---|
| | 1 M Sucrose | (*n*) | Control | (*n*) | Ratio |
| 0.01 M HCl | 1.2 ± 0.2 | (2) | 1.4 ± 0.2 | (2) | 0.9 |
| 0.1 M HCl | 4.5 ± 0.7 | (2) | 6.0 ± 0.0 | (2) | 0.8 |
| 1.0 M HCl | 39.5 ± 0.7 | (2) | 45.0 ± 0.0 | (2) | 0.9 |
| 2.0 M KCl | 2.0 | (1) | 2.4 | (1) | 0.8 |

Details of the experimental procedure are given in the text. Values represent mean currents for (*n*) membranes.

property of the channel itself, so that it cannot be used to test for bulk phase diffusion limitations. A possible explanation is that sucrose decreases the osmotic pressure in the bulk phase and thus decreases water activity in the channel. Zimmerberg and Parsegian (1986) have shown that osmotic stress can close the voltage-dependent anion channel of the outer mitochondrial membrane.

## Single channel proton conductance exceeds 530 pS ($1 \times 10^9$ $H^+$ $s^{-1}$) at saturation

Fig. 4 shows the relationship between bulk phase HCl concentration and proton currents in single gramicidin channels at 59 mV applied potential. There are three distinct segments to the curve in agreement with previous measurements (Eisenman et al., 1980), i.e., a shoulder at 0.01–0.1 M HCl, a nearly 1:1 concentration-to-current relationship between 0.1 and 2.0 M HCl, and no response to added HCl above 4 M.

There were a number of trivial explanations for the saturation we observed at high HCl (> 4 M). First, it was possible that we induced conducting defects in the bilayer itself that dominated the channel population. High acidity can induce spontaneous channels in phospholipid bilayers (Kauffmann and Silman, 1983). To test this, we ran control experiments on GMO/cholesterol bilayers without gramicidin in 2–5 M HCl. Occasionally, distinct current steps up to 2.5 s in duration could be seen, but the conductivity of these defects was a fraction of the conductivity of gramicidin at the same HCl level.

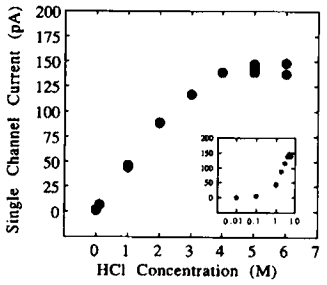


FIGURE 4 The effect of bulk phase HCl concentration on proton currents in gramicidin channels. Each point is the mean amplitude for 50 events at +/−59 mV applied potential. A minimum of two experiments were run at each concentration. Membranes were formed from GMO/cholesterol/hexadecane. The mol ratio of gramicidin to GMO was $1/10^7$ at 4 M HCl and below, and $1/10^8$ above 4 M. The gramicidin content was reduced at high HCl because the open channel frequency increased. Signal output was filtered at 500 Hz except in the 0.01 M HCl treatment where we filtered at 20 Hz due to a low signal-to-noise ratio. Channel amplitude was frequency independent in these experiments. The inset shows a Log [HCl] vs. current plot of the same data.

Second, it was possible that hydrolysis of the bilayer lipid or of gramicidin itself at high HCl caused the observed saturation. To test this, we incubated GMO/cholesterol suspensions in acid for 1 h. Thin-layer chromotography of the lipid showed no hydrolysis at 0.1 M HCl, but significant hydrolysis at 1.0 M HCl and above as indicated by spots that eluted with an oleic acid standard. To quantify GMO hydrolysis, we repeated the experiments for GMO suspensions and measured each spot by flame ionization with an Iatroscan TH-10 detector. After 1 h of incubation in 3–5 M HCl, GMO hydrolysis could be detected (< 10 mol percent). To determine if acid hydrolysis could alter gramicidin conductivity in GMO/cholesterol/hexadecane bilayers, we measured single channel conductivity in bilayers formed from suspensions that had incubated in 5 M HCl for 1 h. We found that there was no significant difference in single channel currents between treated membranes and controls in either 0.1 M HCl or 2.0 M KCl. Thus, there was no irreversible change in the conductivity of gramicidin channels embedded in GMO/cholesterol/hexadecane bilayers despite measurable acid hydrolysis of the lipid in 5 M HCl. This experiment cannot exclude the possibility of a reversible change in the gramicidin channel at 4 M HCl and above.

We conclude that proton flux in the gramicidin channel saturates at ~4 M HCl due to a rate-limiting property of the waters in the channel or due to a reversible change in the channel induced at high acidity. The saturating current under these conditions (Fig. 2) is ~ 140 pA which is equal to $10^9$ protons $s^{-1}$ per channel. This is $10^6$ times faster than the maximum proton current in $F_0$ under physiological conditions, and 75 times greater than the saturated NaCl current measured previously (Hladky and Haydon, 1972).

## Activation energy of proton flux in the gramicidin channel is similar to the activation energy of the L turning defect in ice and much greater than the activation energy of the hopping defect in ice

We measured the Arrhenius activation energy for proton conductance in gramicidin to help identify the rate-limiting mechanism. We chose to make this measurement in 1.0 M HCl because we were interested in the activation energy for proton transfer along the hydrogen-bonded water within the channel rather than in the bulk phase.

A representative experiment is shown in Fig. 5. The

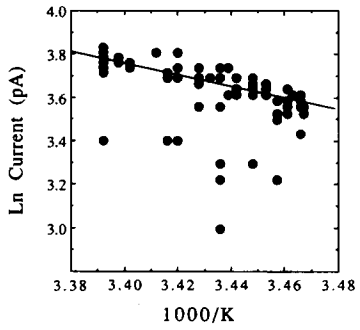


FIGURE 5 An Arrhenius plot of single channel gramicidin A proton currents vs. temperature. Each point represents one event for an experiment conducted at 1 M bulk phase HCl. The wide scatter was due to low amplitude events which we chose to include to avoid systematic errors as the bath cooled from 22° to 15°C. The low amplitude events were randomly distributed with varying temperature. The signal was filtered at 20 Hz.

Arrhenius activation energy for proton flux calculated from four replicate experiments with gramicidin D was 20 ± 3 kJ $mol^{-1}$. We were concerned that this value reflected a selection among possible dimers because gramicidin D is composed of 72% gramicidin A, 9% gramicidin B, and 19% gramicidin C (Glickson et al., 1972). Consequently, we repeated the test with pure gramicidin A. Replicate experiments showed scatter similar to that of gramicidin D and the same activation energy (20 ± 1 kJ $mol^{-1}$). This is much greater than an Ea of 4–6 kJ $mol^{-1}$ for ionic defect mobility in pure ice and in HCl-doped ice (von Hippel et al., 1973; Pines and Huppert, 1985; Onsager, 1973), and it is about equal to an Ea of 22–23 kJ $mol^{-1}$ for turning defects in pure ice and HF-doped ice (Camplin et al., 1978; Eisenberg and Kauzmann, 1969).

## DISCUSSION

The original estimate of proton conductance along putative hydrogen-bonded wires in biological membranes was based on the assumption that proton mobility, $\mu_{H+}$, was similar between the membrane wire and ice (Nagle and Morowitz, 1978). Our data show that this assumption is correct. The mobility of an ion can be defined as

$$\mu = d^2/V\tau, \quad (1)$$

where $d$ is distance traveled in centimeters, $V$ is voltage, and $\tau$ is transit time across the distance (Nagle and Tristram-Nagle, 1983). In the specific case of proton conductance in gramicidin, Fig. 4 shows that current saturates at 140 pA in 4 M HCl and $V$ = 0.059 V. Assuming 1 proton in the channel at any time and no more, $\tau$ would be equal to the inverse of the current or $1.1 \times 10^{-9}$ s. Thus for $d$ = 26 Å across the gramicidin channel (Koeppe et al., 1979), Eq. 1 gives $\mu_{H+} = 1 \times 10^{-3}$ $cm^2\ V^{-1}\ s^{-1}$. This is intermediate between the mobilities of the hopping defect in ice ($\sim 5 \times 10^{-3}\ cm^2\ V^{-1}\ s^{-1}$) and the turning defect ($\sim 5 \times 10^{-4}\ cm^2\ V^{-1}\ s^{-1}$) in ice (Nagle and Tristram-Nagle, 1983).

From our data we cannot tell which of these two obligatory mechanisms is rate limiting in gramicidin. The activation energy that we measured for proton conductance (20 kJ $mol^{-1}$) is close to that of the turning defect in ice (22–23 kJ $mol^{-1}$), suggesting a similar rate-limiting step. However, in our view it is improbable that the gramicidin water wire is as rigid as crystalline ice as would be required for this to be true. We postulate, instead, that bent or stretched H-bonds between waters constrained in the channel may increase the Ea for the hopping defect in gramicidin relative to ice as predicted by ab initio quantum mechanical calculations (Scheiner, 1985).

Hydrogen-bonded water molecules nested within clusters of transmembrane proteins could provide a pathway for proton currents in biological channels (Onsager, 1967; Hoppe and Sebald, 1984; Schulten and Schulten, 1985; Deamer and Nichols, 1989). In bacteriorhodopsin, for example, a recent structural model at 3.5 Å resolution strongly suggests that waters in a narrow pore play a role in proton delivery to the chromophore (Henderson et al., 1990). A test of this postulate for the $F_0$ subunit is that proton transfer along the water wire must be fast enough to permit sufficient proton current for ATP synthesis. Our experiments at 59 mV applied potential indicate that the protonic current in the gramicidin water wire saturates at 140 pA (4 M HCl) which is equal to $1 \times 10^9$ protons $s^{-1}$. This is $10^6$ times faster than the proton-transfer rate required for ATP synthesis and $\sim 10^3$ times faster than the maximum protonic current in $CF_0$ (Lill et al., 1987). Clearly the water wire itself is kinetically competent provided adequate proton supply from the bulk phase. Moreover, protonic conductance by the gramicidin wire is intermediate between conductances of channels formed at interfaces between α helices (Table 2). This reinforces the plausibility of water wires at similar interfaces in $F_0$.

Proton supply to a putative water wire is a separate question that becomes important in the physiological pH range. Our current-voltage data (Figs. 2 and 3; see also Eisenman et al., 1980), and our hydrogen-deuterium isotope ratio data show that proton flux along the gramicidin water wire is limited by supply from the bulk phase at pH 2 or greater. It is probable, therefore, that

TABLE 2 **Comparison between $H^+$ currents in the gramicidin channel and $H^+$ currents at interfaces between leucine-serine α helices**

| | $(LSSLLSL)_3$ | Gramicidin | $(LSLLLSL)_3$ |
|---|---|---|---|
| $G_{H^+}$ ($pS$) | 900 | 530 | 120 |
| $G_{H^+}/G_{K^+}$ | 4.2 | 45 | >58 |
| HCL concentration at saturation ($M$) | — | 4 | 3 |
| Channel diameter ($Å$) | 8 | 2–4 | 1 |

Values for gramicidin are from this report except for the numerator in row two (Haydon and Hladky, 1972) and the gramicidin channel diameter (Koeppe et al., 1979). All values for the two synthetic polypeptides were from Lear et al. (1988) and DeGrado and Lear, (1990). $(LSSLLSL)_3$ and $(LSLLLSL)_3$ refer to polypeptides that contain three repetitions of the leucine (L), serine (S) sequence shown. Proton conductances in the first row are for 150 mV applied potential and 0.5 M HCl in the bulk phase. Conductance ratios in the second row are for HCl and KCl at bulk phase concentrations where the current is saturated at 100 mV, except for $(LSSLLSL)_3$ where Lear et al. (1988) did not specify conditions of the experiment. The $H^+/K^+$ conductance ratio for $(LSLLLSL)_3$ uses the detection limit of 5 pS in those experiments as the denominator. Thus, the true conductance ratio may be much greater.

proton supply to the $F_0$ channel from the thylakoid lumen (pH 5) and from the cytosol bathing the mitochondrial membrane (pH 7.5) would limit proton flux. If the channel target is a water wire, would this supply be adequate to account for ATP synthesis? To address this question, we note that the proton motive force in chloroplasts and mitochondria is ~200 mV. Fig. 2 shows that at pH 2 and 200 mV applied potential, the water wire current is 1.1 pA or $6.9 \times 10^6$ $H^+$ channel$^{-1}$ $s^{-1}$. The proton current in a population of gramicidin channels appears to decrease linearly with proton concentration below 0.01 M (Eisenmann et al., 1980), therefore at pH 5 (the pH of the chloroplast thylakoid lumen; Althoff et al., 1989) proton flux along the gramicidin water wire would be $6.9 \times 10^5$ $H^+$ channel $^{-1}$ $s^{-1}$. This is sixfold greater than proton flux required for the maximum rate of ATP synthesis in $CF_0CF_1$ of intact chloroplasts (1200 $H^+$ channel$^{-1}$ $s^{-1}$).

Adequate proton supply to a water wire in the mitchondrial ATP synthase is less certain. Using the same logic as above, proton supply from the cytosol at pH 7.5 would be only 20 $H^+$ channel$^{-1}$ $s^{-1}$. This suggests three possible explanations: a much wider channel mouth, a much slower rate of ATP synthesis per enzyme, or additional mechanisms of proton supply to the mitochondrial ATP synthase. One intriguing possibility is that in mitochondria where $\Delta\psi$ drives flux, hydrolysis of water at the channel mouth could be a major source for protons as is true for protonophores such as S-13 (Kasianowicz et al., 1987). This explanation could only be true if the linear relationship between dilute bulk phase proton concentration (<0.01 M) and protonic current in gramidicin channel populations were due to channel open time or open frequency rather than single channel amplitude.

Finally, is a water wire consistent with the extreme proton selectivity observed in $CF_0$? To answer this question, it is important to note that the proton selectivity observed by Lill and co-workers (1987) is not due to an anomalously high proton conductance (~1 pS compared with 5–150 pS for $Na^+$ and $K^+$ in their respective channels; Hille, 1984), but rather it is due to the apparent failure of $Na^+$ or $K^+$ to permeate the channel even at 300 mM electrolyte concentration. Thus, $CF_0$ effectively excludes all ions except protons. Exclusion of this sort could be due to either of two mechanisms: rejection of ions larger than the diameter of the channel (the channel acts as a sieve); or specific binding of the desired ion to a site (an energy well) and failure of that site to bind other ions (Tsien et al., 1987; Eisenman and Dani, 1987).

With our data we can reject the possibility of a continuous transmembrane water wire in $F_0$ analogous to that in gramicidin. That is, such a channel must be at least 2.5 Å in diameter at all points to accommodate water molecules, and would therefore fail to exclude $Na^+$ or $K^+$ flux because their diameters are significantly <2.5 Å. A proton-selectivity ratio on the order of 100/1 would be expected rather than $10^7$/1 as in $CF_0$ or even $10^3$/1 as observed for the $Na^+$ ATP synthase (Laubinger and Dimroth, 1989). In contrast, water strands that extend only part way across the membrane, analogous to the waters postulated to lead to the chromophore in bacteriorhodopsin (Henderson et al., 1990), are consistent with extreme proton selectivity for two reasons. First, to permeate a channel lined with a single strand of waters (such as gramicidin) alkali cations must push waters through the channel (Levitt, 1984). A water-impermeant barrier (e.g., $Arg_{210}$ of subunit *a* and $Asp_{61}$ of subunit *c* in *E. coli*, $F_0$) could prevent displacement of waters and would preclude $K^+$ or $Na^+$ currents in $F_0$. Second, amino acids at the $F_0$ reaction center could be specific for protons. For example, the $-NH_2$ groups of arginine and histidine readily form covalent bonds to protons but they have very low affinities for alkali cations (Williams, 1988).

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